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THE NEMATOCYST TOXIN OF *METRIDIUM*[1]

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At the beginning of the century, Richet (Richet and Portier, 1936) separated four pharmacologically active substances from the tissues of the sea anemone *Actinia* (= *Metridium* [Hand, 1954]). Since that time a great deal of effort has been directed toward the investigation of toxic materials occurring in coelenterates. It is now generally accepted that Richet's extracts were mixtures of several substances (Welsh, 1961), and a variety of compounds have been suggested as possible components of coelenterate toxins. Among the substances reported in toxic extracts of coelenterate tissues are histamine (Mathias, Ross and Schacter, 1960), tetramethylated ammonium bases (Welsh and Prock, 1958), serotonin (Welsh, 1960), polysaccharide substances (Phillips, 1956; Martin, 1966), and proteins of various molecular weights (Phillips and Abbott, 1958; Kline and Waravdekar, 1960; Lane, 1961; Blanquet, 1968). Several methods for obtaining isolated nematocysts have been described (Phillips, 1956; Lane and Dodge, 1958; Yanagita, 1959). Some investigators, however, have continued to use a variety of tissues, and in some cases entire animals, as a source of material presumed to originate in the nematocysts. The situation has been further complicated by the wide variety of animals used as sources of test material and for bioassay.

The pharmacological effects of coelenterate toxins have been investigated on a variety of physiological preparations including frog muscle, rat uterus and guinea pig ileum (Mathias *et al.*, 1960); mice (Lane, 1961; Martin, 1966); canine cardiovascular system (Hastings, Larsen and Lane, 1967); molluscan heart (Welsh, 1956); and crustacea (Lane, 1961; Blanquet, 1968). These studies have provided valuable clues as to the possible nature of coelenterate toxins. Unfortunately, it has been difficult to correlate pharmacological effects with specific substances known to be present in coelenterates, and the reaction of different assay preparations to the same compound have varied widely (*cf.* Welsh, 1956; Mathias *et al.*, 1960; Hastings *et al.*, 1967).

The purpose of the following study was to investigate the toxicity of material released upon discharge of isolated nematocysts from *Metridium* and to classify the principle active components of the toxin. Serotin, quaternary amines and indoles which are repeatedly mentioned in connection with cnidarian toxicity, were given particular attention.

Materials and Methods

Sea anemones, *Metridium senile senile* Linnaeus (1767), were obtained from the vicinity of St. Andrews, New Brunswick. Isolated nematocysts were obtained by the method of Blanquet (1968). Five or six anemones (approximately 15 g live wet weight per anemone) were placed in 200 ml of 1 *M* sodium citrate, and prodded with a glass rod to induce the expulsion of acontia. After five to ten

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minutes, the animals were removed from the citrate, and the acontia were allowed to incubate for 12 hours at 4° C. This treatment results in the extrusion of undischarged nematocysts from the acontia threads. The citrate suspension was then passed through a nylon mesh screen (Nytex 64, B. and S. H. Thompson Ltd., Montreal) with the aid of suction to remove the acontia threads, centrifuged in the cold (4° C) at 480 × *g* for one hour to collect the nematocysts, and the supernatant fluid was decanted. The isolated nematocysts were examined microscopically in order to determine the percentage undischarged. The nematocysts were discharged by re-suspending in distilled water followed by incubation for 12 hours at 4° C. At the end of this time, a second examination was made for undischarged nematocysts. The nematocyst-fluid mixture was centrifuged in the cold (0° C) for 40 minutes at 12,100 × *g*, and the resulting supernatant lyophilized in a Virtis micro freeze-drying apparatus. The apparatus was kept refrigerated at 4° C or below during the lyophilization process. The resulting lyophilate was termed "crude toxin extract." In some cases, the pellet from the final centrifugation was homogenized in a chilled Potter-Elvejhem apparatus, centrifuged, and the supernatant lyophilized. The lyophilate so obtained was termed "crude pellet extract."

Bioassay of extracted material was performed on crayfish, *Orconectes virilis* or *O. propinquus* obtained from streams in the vicinity of Toronto, Ontario, and from a local supply house. These species were used interchangeably, as they responded identically to crude toxin extract in early experiments.

Lyophilates of material to be assayed were dissolved in distilled water to give the desired concentration, and were injected into the hemocoel of the test animals via the base of the third walking leg. Bovine serum albumin (BSA), sea water, and sodium citrate were initially employed as controls. In later experiments, BSA only was used.

LD_{50} values were determined as follows: Groups of test animals (*O. virilis* or *O. propinquus*) were injected with doses of crude toxin extract based on the wet weight of the crayfish. Six animals were used at each dose level, and a sufficient variety of dosages used to provide at least five points in plotting the dose-response line. Deaths occurring within 24 hours were considered to have resulted from injected material. Criteria of death were lack of movement when probed and loss of tonus in the chelipeds. No surviving animals were re-used. Response data were analyzed by plotting the logarithm of dose against the percentage mortality, and fitting a straight line by regression (Snedecor and Cochran, 1967). A dose calculated to produce 50% mortality was then determined with confidence limits at the 95% probability level.

Potency of various extract fractions, relative to that of the whole crude extract, was calculated by the single dose method of Bliss (1952). In this method the mean response of two groups of test animals to a single dose level of the fraction being assayed is compared to the expected response based on the line used to calculate the LD_{50}.

Crude pellet extracts were examined for toxicity by injecting doses ranging from 100 to 750 mg/kg live body weight into six-animal test groups of crayfish *O. virilis*.

To determine the effects of heating, prolonged hydration, and repeated lyophilization, samples of crude toxin extract were rehydrated, heated at 55° C for 30

minutes and assayed on two groups of crayfish as described above. Additional samples were rehydrated and incubated at 4° C for 48 hours, after which they were assayed as for the heat-treated material. Samples of crude toxin extract were rehydrated and immediately lyophilized. The lyophilate was rehydrated and assayed as described above.

Studies of comparative toxicity were conducted on two ten-animal test groups of the fiddler crabs *Uca pugilator* and *Sesarma reticulatum* from the vicinity of Panacea, Florida, and on two six-animal test groups of the crayfish *Procambarus econfinae*, obtained from a commercial supplier in the southeastern United States. The results of these studies were compared with data from toxicity studies using *O. virilis* and *O. propinquus*.

Since protein was suspected of being responsible for part or all of the toxic activity of the crude toxin extracts, protein content of each batch was determined by the Folin method described by Lowry, Roseborough, Farr and Randall (1951), using BSA (Sigma Chemicals) as the protein standard. As a check on the accuracy of the Folin method, nitrogen content of duplicate samples of crude toxin extracts was determined by the micro-Kjeldahl method described by Meites and Faulkner (1962).

In an attempt to concentrate the toxic material, dialyzed extracts were prepared by rehydrating 200 mg samples of crude toxin extract in 20 ml distilled water and dialyzing against a single 3 l volume distilled water. The dialysis system was held for 48 hours at 4° C, after which the material in the dialysis tubing was lyophilized. Cellulose dialysis tubing (Sargent S-252275A) was used. Approximate pore size was determined by dialysing solutions of glucose, sucrose, and raffinose, and analyzing for carbohydrate passage through the tubing. Protein determinations were made on each of the dialyzed extracts using the Folin method described above. The dialyzed extracts were tested for toxic activity by injecting two groups of six crayfish with doses of extract based on the body weight of the individual crayfish.

Additional 200 mg samples were rehydrated in 5 ml distilled water and dialyzed for 48 hours against a single 20 ml volume distilled water. The dialyzable and undialyzable fractions were then assayed for total nitrogen content using the micro-Kjeldahl method described above. In order to correlate dialyzable nitrogen with non-protein nitrogen, residual nitrogen was determined in solutions of 100 mg crude toxin extract which had been deproteinized by the addition of zinc sulfate and barium hydroxide according to the method of Meites and Faulkner (1962).

Further 100 mg samples were rehydrated in 2 ml distilled water and dialyzed against 20 ml distilled water for 48 hours. Dialyzable and undialyzable materials were lyophilized. These preparations were assayed on the crayfish *Procambarus econfinae*.

As a potential aid in identifying the components of the toxic material, ultraviolet absorption spectra were determined on crude toxin extract, dialyzable material and undialyzable material using a Zeiss PMQ II spectrophotometer.

Further information on the composition of the crude toxin extract and dialyzed preparations was obtained using paper chromatography on Whatman #1 filter paper. 500 μg samples of unhydrolyzed crude extract and dialyzed material were used. One dimensional chromatograms were performed using butanol-acetic acid-

water (10:3:1), which proved to be more satisfactory than other solvent systems. Acetone-water (8:2) was employed as the second solvent system for two dimensional chromatograms. Dried chromatograms were scanned with ultraviolet light (Mineralight SL 2537, short wave filter) to detect absorbing or fluorescing spots. Following this, one of a variety of detection reagents was used: 0.2% ninhydrin or copper sulfate for amines, Ehrlich's reagent for indoles, Nessler's reagent for carboxylic acids, acetylacetone and p-dimethylaminobenzaldehyde for hexosamines, 1-nitroso-2-naphthal for 5-hydroxyindoles, potassium dichromate and 40% formalin for aromatic amines (Dawson, Elliot, Elliot and Jones, 1959), and Dragendorf's reagent for the detection of quaternary ammonium bases (Bregoff, Roberts and Delwiche, 1953). In addition, serotonin content was determined by the method of Udenfriend, Clark and Neissbach (1955), using serotonin creatinine sulfate (British Drug Houses) as the standard.

Since the results of paper chromatography and the presence of dialyzable nitrogen compounds made the presence of amines a strong possibility, derivatives of crude toxin extracts were prepared for possible melting point characterization. Amines were prepared as actyl derivatives by reacting crude extract with acetic anhydride, as benzenesulfonyl derivatives by reaction with benzenesulfonyl chloride, and as picrates by reaction with picric acid. Amide derivatives of carboxylic acids were obtained by reaction with thionyl chloride and subsequent coupling with ammonium hydroxide. Melting points were determined by placing a small amount of the derivative into capillary tubes with one end sealed, and heating in n-butyl phthalate until melting occurred. Two melting point determinations were made for each derivative obtained.

The amino acid content of a combination of batches of crude toxin extract was determined with a Beckman amino acid analyzer, for comparison with studies which have reported high concentrations of aspartic acid (Lane, 1961), or glutamic acid (Lane and Dodge, 1958), or the absence of tyrosine (Phillips, 1956).

Crude toxin extracts were tested for carbohydrate materials by dissolving 100 mg of extract in 1 ml distilled water, and reacting with alpha-naphthol and sulfuric acid (Molisch test). Lipid content of crude toxin extracts was determined gravimetrically by extracting 100 mg samples with chloroform using the Sperry-Brand technique (Ackermann and Toro, 1953).

Reagent grade chemicals were used throughout this study.

Results

Microscopic examination at intermediate stages in the preparation procedure revealed that the majority of nematocysts were of three types: basitrichous anastigophores, microbasic mastigophores, and microbasic amastigophores (terminology of Hand, 1961). Samples taken prior to incubation in distilled water had 1–10% of the nematocysts discharged. After incubation in distilled water samples were 72–92% discharged. Very little cell debris was noted in pre- or post-discharge samples. Batches of crude toxin extract were all toxic, although the degree of toxicity varied widely. No deaths occurred among control animals in any of the bioassays performed in this study, including those injected with sodium citrate at dose levels up to 750 mg/kg. Rehydration and incubation for 48 hours at 4° C was found to reduce the toxicity of crude toxin extract (relative potency less than

0.338). Heating for 30 minutes at 55° C also lowered the toxic activity of the extract (relative potency less than 0.093). Repeated lyophilization had no measurable effect on the activity of crude toxin extract.

Crude pellet extracts

Three batches of crude pellet extract were not lethal to any animals at dosages up to 750 mg/kg live body weight.

Results of dialysis

Calibration of dialysis tubing with various sugars revealed that the tubing used would pass material with molecular weight of 360 or less, but would not pass substances with molecular weight of 540 or more (a more precise estimate of the exclusion size of the tubing was not attempted, since this range would almost certainly prevent proteins from passing across the membrane).

TABLE I

Effect of dialysis and precipitation by barium hydroxide on nitrogen levels of material released by discharge of isolated nematocysts of Metridium senile

Sample Number	Total nitrogen (μg/mg extract)	Dialysable nitrogen (μg/mg extract)	Unprecipitable nitrogen (μg/mg extract)
1	2.00	0.70	0.74
2	4.20	2.00	2.40
3	1.90	0.90	0.92
4	0.90	0.24	0.29

Samples of crude toxin extract which had been dialyzed against a single 31 volume of distilled water were found to have increased protein concentrations of 18–34 fold. Bioassay, however, failed to demonstrate any increase in toxicity. Folin protein determination of these preparations revealed that about half of the material supposed to be protein in crude toxin extracts was dialyzable. Nitrogen determinations of dialyzable and undialyzable fractions showed that 30–50% of the nitrogen present in the crude toxin extract was dialyzable. In addition, the dialyzable nitrogen was found to correspond closely to the nitrogen remaining after crude toxin extracts had been deproteinized by barium hydroxide and zinc sulfate. These results are summarized in Table I. Crude toxin extracts, when deproteinized, still gave a blue color when treated with the Folin reagent. This reactivity disappeared, however, after dialysis.

Both dialyzable and undialyzable material were found to have toxic activity when assayed on *P. econfinae*, and in this case the undialyzable material had actually increased in toxicity relative to crude toxin extract assayed on the same species. The relative potency of the undialyzable material was 5.94, and the relative potency of the dialyzable material was 0.705.

Differential toxicity

As it seemed possible that *Procambarus econfinae* had a different sensitivity to the toxin preparations than *Orconectes propinquus*, the potency of crude toxin extract in *P. econfinae* was determined relative to *O. propinquus* and was found to be 2.56.

Fiddler crabs *Uca pugilator* and *Sesarma reticulatum* were injected with samples of extract at the LD_{50} level for *O. propinquus*. None of the animals in a 20-animal test group (each species) had shown any response (paralysis, autotomy, tetany or death) 24 hours after injection.

TABLE II

Amino acid analysis of material released on discharge of isolated nematocysts of Metridium senile

Amino acid	Concentration in 1.00 g sample (μg)
Aspartic acid	204.022
Threonine	91.035
Serine	128.205
Glutamic acid	250.047
Proline	77.970
Glycine	103.500
Alanine	115.344
Valine	82.485
Methionine	41.571
Isoleucine	70.740
Leucine	114.363
Tyrosine	82.355
Phenylalanine	69.300
Total	1470.937
Unknown	approximately equimolar with proline

Results of paper chromatography

Paper chromatograms of crude toxin extract, dialyzable material, and undialyzable material all showed two spots with r.f. values of 0.20 and 0.42. The spots were less intense in the undialyzable fraction, but were distinct. In addition, a spot was observed at the point of application on chromatograms of crude and undialyzable material. This spot was ninhydrin positive, gave a blue Ehrlich reaction, and absorbed ultraviolet light. No further separation was obtained when two dimensional chromatograms were run. Both mobile spots gave a light blue fluorescence in ultraviolet light, a blue ninhydrin reaction, yellow Ehrlich reaction, reacted with copper sulfate, and gave a yellow-orange fluorescence in ultraviolet light when sprayed with potassium dichromate-40% formalin. The spot with r.f. = 0.20 produced a brown-orange spot when treated with Nessler's reagent. No reaction was observed with Dragendorf's reagent, 1-nitroso-2-naphthol, or acetylacetone-p-dimethylaminobenzaldehyde. Six batches of toxin run on the same chromatogram gave identical results.

Results of other characterization procedures

Ultraviolet absorption spectra show a slow decline from 200 mμ to 350 mμ without sharp peaks. This is probably due to inadequate purity of samples. Similar results were obtained for both crude and dialyzed materials.

Of the melting point derivatives obtained, only the picrate and benzenesulfonyl derivatives were useful. The other preparations decomposed in the region of 230° C without producing distinct melting points. The picrate derivative melted at 162–164° C, while the benzenesulfonyl derivative remained stable at temperatures up to 300° C.

Results of amino acid analysis on a combination of three batches are summarized in Table II. The quantities given were contained in 1.00 g crude toxin extract. The sum of these quantities is 1470 μg total amino acids. The amount of protein that should have been present in the analyzed material is 5392 μg, based on the Folin protein determinations of the original batches. If average concentrations (West and Todd, 1961, pages 280–281) are assumed for the amino acids not shown in the analysis, the quantity of amino acids is about one-third that of the Folin reactive material.

All batches of crude toxin extract were found to contain no carbohydrate material by the Molisch test, and only traces (less than 0.2%) of lipids by chloroform extraction.

Discussion

A significant quantity of the total nitrogen of nematocyst contents preparations was dialyzable. The dialyzable material reacts in a manner similar to that of protein when exposed to the Folin reagent. It is, however, very unlikely that the dialyzable substance is a protein because of the small particle size involved (molecular weight less than 540). The Folin method for protein estimation is based on reactions with tyrosine and peptide bonds. It seems probable that other aromatic amines might also react with the reagent, and this could account for the reduction of Folin reactive material in the dialyzed preparations. The amino acid analysis substantiates this explanation, because it indicates a protein level in crude toxin extracts which is about one-third of that indicated by the Folin method.

The possibility of the dialyzable substance being a small peptide was considered. Such a peptide would have to have a molecular weight of less than 540, which means that it could be composed of a maximum of seven amino acids (heptaglycine, mol. wt. 525). If a peptide such as this were present, it would react with the Folin reagent, and the component amino acids would be counted as part of the amino acid analysis of crude toxin extract. The total amino acids, however, accounted for only one-third of the Folin reactive material, and barely accounts for the total precipitable nitrogen. This makes the possibility of an unprecipitable, dialyzable amino acid compound unlikely, and indicates that there must have been present in the crude extract some substance other than amino acids capable of reacting with the Folin reagent.

The toxicity of crude and dialyzed preparation in *Procambarus econfinae* suggests that there are at least two toxic moieties, one dialyzable and the other undialyzable. If there are, in fact, two toxic fractions, there is a possibility of syner-

gistic effects, such as those occurring in certain snake venoms (Dimitrov and Kankonkar, 1968). Recombining dialyzable and undialyzable materials failed to increase the activity, but this does not negate the possibility of synergism, since prolonged hydration was shown to cause some change in the toxic material. The fact that heating, as well as prolonged hydration, also abolishes toxic activity of the extracts, together with the amino acid analysis, indicates that the non-dialyzable fraction is proteinaceous.

The results of chromatography also indicate that a proteinaceous substance and dialyzable nitrogen compounds were present in the extracts. A non-mobile spot at the point of application on chromatograms of crude and undialyzable material is indicative of a protein-like substance in these materials. The reaction of the spot with ninhydrin and Ehrlich's reagent, and ultraviolet absorption are all characteristic of proteins. The two mobile spots were observed in crude, dialyzable, and undialyzable material. Their occurrence in the undialyzable fraction is not altogether surprising, since at equilibrium in the dialysis system there would theoretically be equal concentrations of dialyzable materials on both sides of the dialysis membrane, assuming no electrochemical effects occurred.

The blue ninhydrin reaction, combination with copper sulfate, and yellow Ehrlich reaction are all indicative of amines. Sky-blue fluorescence in ultraviolet light and reaction with potassium dichromate-40% formalin are indicative of aromatic amines. The reaction of one of the spots with Nessler's reagent indicates that this compound is carboxylated. It is unlikely that these compounds are indoles, serotonin analogs, or tetramethylated ammonium compounds, since no reaction was observed with reagents specific for these substances. Red colors with acetylacetone-p-dimethylaminobenzaldehyde were also not obtained, making the presence of hexosamines unlikely.

The formation of picrates is indicative of amines in general, while benzenesulfonyl compounds are formed by primary and secondary amines only. The melting points of these derivatives closely resemble the derivatives of certain intermediate compounds in tryptophan metabolism (*e.g.*, kynurenine, quinaldic acid), fused ring or polysubstituted aromatic compounds (*e.g.* naphthylamine) and histamine. The latter is noteworthy, in view of a suggestion by Welsh (1956) that, because of similarities in its pharmacology, histamine or a similar compound may be involved in toxic reactions to *Metridium* nematocysts.

Glutamic and aspartic acids were the most abundant amino acids in the hydrolysate of crude toxin extract. These findings are in agreement with those summarized by Picken and Skaer (1966).

It is interesting that many of the compounds which have been held by other workers to be responsible for nematocyst toxicity were not found in this study. Welsh and Prock (1958) reported a variety of quaternary ammonium compounds in extracts made from whole animals or tentacles of *Metridium dianthus*. Phillips (1956) reported the presence of serotonin in *Metridium senile* nematocysts, and Welsh (1960) found high concentrations of this substance in tentacles of the same species. Phillips and Abbott (1957) later noted, however, that the concentration of serotonin fell steadily as the toxin was purified (the nature of the purified toxin obtained by Phillips has not been published). Lentz and Wood (1964), using histochemical detection methods, have found serotonin both inside the nematocyst

and the surrounding cnidoblast of *Metridium*. No indication was obtained in the present study of the presence of serotonin in either tentacles or crude toxin extract, nor was any indication found of the tetramethylated ammonium bases which have been reported.

The occurence of differential toxicity dependent upon the species of animal used for bioassay suggests that many seemingly contradictory studies may, in fact, be compatible. Welsh and Prock (1958, page 558) reported that extracts of *Metridium dianthus* tentacles contain "factors which, in considerable dilution, produce spontaneous autotomy of legs, followed by paralysis and death [in *Uca pugilator*]." Phillips and Abbott (1957) reported that extracts of isolated nematocysts were pharmacologically active in *Littorina planaxis*. In contrast, Mathias *et al.* found no pharmacologically active substance in macerated tentacles of *Metridium senile* when assayed in isolated organ preparations from a variety of vertebrates. In view of the highly variable response of different species of animals to the same extract in the present study, it is reasonable to suppose that the different findings actually reflect the difference in the assay organisms, rather than in the substances being assayed. Indeed, Welsh (1956) reported differences in pharmacological activity of tetramethylammonium, which mimics the action of *Metridium* toxins assayed on *Uca pugilator*, but has the opposite effect on *Hemigrapsus nudus*.

Although research on Scyphozoan nematocyst toxins has been comparatively scanty the available information suggests that there are similarities between Scyphozoan and Anthozoan toxins. Welsh (1956) found evidence of an amine in *Cyanea* tentacle extracts which has excitory action when assayed on the isolated heart of the horse clam *Schizothaerus nuttallii*. In a later paper, Welsh and Prock (1958) reported the presence of tetramethylammonium, homarine, trigonelline, and γ-butyrobetaine in tentacles of *Cyanea*, but pure samples of these compounds did not have the same pharmacological activity as *Cyanea* tentacle extracts when assayed on *Uca pugilator*. Barnes (1966) has reported on the occurrence and pharmacology of the Irukandji carybdeid, *Chironex fleckeri*, and *Chiropsalmus quadrigatus* which are a serious health hazard in tropical waters. The symptoms produced are skin wheals, edema, deep tissue necrosis, and cardiac arrest. The lack of paralysis and general organ pathology suggest that the toxic agent(s) may be similar to those found among the Anthozoa.

The obvious discrepancies in reported toxin compositions among the various studies are probably due to tissue contaminants in various preparations. The little work which has been done with isolated nematocysts seems to indicate that the toxin of a particular species of cnidarian is fundamentally simple, and there is, in fact no direct link between any of the substances reported in tissue extracts and the toxicity of nematocyst contents. The results of this study indicate that the toxin of *Metridium senile* is also simple, in that it may be composed of a protein and one or two aromatic amines, which previously have not been reported.

Summary

1. Material discharged by isolated nematocysts of *Metridium senile senile* Linnaeus (1767) has been found to be toxic to crayfish *Orconectes virilis* Hagen (1870), *O. propinquus* Girard (1852), and *Procambarus econfinae* Hobbs (1942).

2. The toxin appears to be composed of a proteinaceous, undialyzable fraction, and a dialyzable fraction containing two aromatic amines.

3. The amines are probably fused or polysubstituted ring compounds, one of which is carboxylated.

4. The crude toxin has varying potency, depending upon the species of assay animal used.

5. With the possible exception of histamine, none of the compounds previously suggested as possible components of the nematocyst toxin were found.

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